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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Genomic profiling of Parkinsonian substania nigra pars compacta; alterations in ubiquitin-proteasome, heat shock protein, iron-regulated proteins, cell adhesion/ celular matrix and vesicle trafficking genes

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GENOMIC PROFILING OF PARKINSONIAN SUBSTANTIA NIGRA PARS COMPACTA; ALTERATIONS IN UBIQUITIN-PROTEASOME, HEAT SHOCK PROTEIN, IRON-REGULATED PROTEINS, CELL ADHESION/CELULAR MATRIX AND VESICLE TRAFFICKING GENES.

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Running title; Genomic profiling of parkinsonian substantia nigra pars compacta

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Summary

Parkinson disease (PD) is the most common cause of dementia in last period of humans life. With known methods, the diagnosis of PD is definitively made too late for a successful therapy, but no method for early diagnosis is known.

It is desirable to diagnose PD at an early stage and the present invention represents an early diagnostic method for PD in humans before the symptoms appear. The suggested method includes the determination of the gene expression pattern from up to 137 genes by extraction of RNA from biological material, preferably blood samples or biopsy samples of skin. The gene expression pattern will be determined via comparison to the expression of PD patients and healthy subjects. The collected data should be similar to the pattern described in table 5 and 6 in order to define the subject as PD patient.

INTRODUCTION

Neuropathological and neurochemical studies on substantia (SN) from Parkinson's disease (PD) brains and its animal models, have established several pathogenic processes at the time of neuronal death, though the etiology of the disease remains elusive. Potential contributing factors include ongoing selective oxidative stress (OS) resulting from mitochondrial dysfunction, auto-oxidation or enzymatic (monoamine-oxidase) oxidation of dopamine (DA) and excessive iron accumulation in the SN pars compacta (pc) (Riederer et al., 1989; Youdim et al., 1993; Gotz et al., 1994; Jenner and Olanow, 1996; Olanow and Youdim, 1996; Youdim and Riederer, 1997; Jenner, 1998). Specifically, redox-active iron has been observed within the melanin containing neurons that selectively die and in the rim of Lewy body, the morphological hallmark of PD. Lewy body is composed of lipids, aggregated alpha (α)-synuclein (concentrating in its peripheral halo) and ubiquitinated, hyperphosphorylated neurofilament proteins (Jellinger, 2003). A number of recent studies (Ostrerova-Golts et al., 2000; Ebadi et al., 2001; Turnbull et al., 2001) have shown that α-synuclein forms toxic aggregates in the presence of iron and this is considered to contribute to the formation of Lewy body via OS. Misregulation of brain iron metabolism has taken center stage in neurodegenerative diseases since a significant number of mutated iron metabolism genes have now been shown to be directly involved in neurodegeneration (Felletschin et al., 2003; Youdim and Riederer, 2004). Thus, iron redox status constitutes a pivotal factor contributing to the extent of protein misfolding and aggregation in the ageing and disease affected brain.

Defects in ubiquitination and proteasomal protein handling are common features in PD and other chronic neurodegenerative diseases such as Alzheimer's disease (AD), Amyotrophic Lateral Sclerosis (ALS) and Huntington disease, and in ageing (Ciechanover and Brundin, 2003; Dawson and Dawson, 2003). This may, in turn, lead to impairment in several cellular processes linked to ubiquitination such as cell cycle, processing and regulation of transcription, intracellular trafficking, signalling pathways and degradation of normal and damaged intracellular proteins (Ciechanover and Brundin, 2003). Consistent with this is the accumulating evidence pointing to a crucial role for protein misfolding and aggregation

into protein inclusions in sporadic PD, which constitutes the most common form of the disease. A loss of 20S proteasome alpha-subunits (McNaught et al., 2002; McNaught et al., 2003) and reduction in the activity of the 26/20S proteasome system in SNpc of sporadic PD (McNaught et al., 2003) has also been reported.

Both familial and sporadic forms of PD primarily converge in impairment of protein handling, catabolism and in oxidative-stress damage. In support of this, three apparently independent gene mutations in α-synuclein, parkin and ubiquitin C-terminal hydrolase-L1 (UCHL-1), which are capable of impairing the activity of the ubiquitin-proteasome system (UPS), have been described in rare forms of hereditary PD (Dauer and Przedborski, 2003). More recently, recessive mutations in DJ-1 were proposed to play a role in cellular response to oxidative stress (Bonifati et al., 2003). This suggests a common pathogenic base in both idiopathic and genetic forms of the disease. Accordingly, the particular vulnerability of the parkinsonian dopaminergic neurons of the SNpc to OS, together with a failure of the UPS to adequately remove abnormal proteins, have been suggested to constitute the basis of the etiopathology of sporadic PD. However, none of these genes have been demonstrated to be mutated in sporadic PD, which constitutes more than 95% of total PD cases. Even more confounding is the findings that deletions in Parkin or its suggested catalytic substrates and in other gene-linked PD, do not reproduce the disease phenotype, since they don't lead to specific dopaminergic cell loss. Furthermore, Lewy bodies with aggregated ubiquitinated proteins are beginning to be viewed as a defensive measure aimed at removing toxic misfolded damaged proteins, rather than a mere general site for precipitated misfolded proteins (Hashimoto et al., 2004; Tanaka et al., 2004).

Among the neurotoxins used to induced parkinsonism in animals, MPTP best replicates many of the neurochemical and anatomical characteristics of the Parkinsonian syndrome in rodents, primates and other species (Dauer and Przedborski, 2003). In spite of certain limitations intrinsic to this model, namely lack of progressive nature and Lewy bodies (inclusion bodies), much has been learned about the molecular events that lead to dopaminergic neurodegeneration. For example, the MPTP model has recently been employed to assess differential gene expression changes in the midbrain of mice

(Grunblatt et al., 2001), to obtain a more global picture of the series of events occurring during degeneration. The median density microarray employed in the study contained around 1200 genes. Thus, a limited picture of the differential gene expressions that may be altered was observed. Nevertheless, the study has demonstrated alterations in genes related to oxidative-stress, inflammation (e.g. cytokines, prostaglandins), nitric oxide, glutamate excitotoxicity and neurotrophic factors pathways, which were up or down regulated. Additional gene pathways, not described previously, including cell cycle and iron metabolism regulation, apoptosis, intermediary metabolism and signal transduction have also been observed. Because of the uncertainty of which of the several factors constitutes the direct trigger in the etiopathology of PD neurodegeneration, this disease may be considered a syndrome with different etiologies, rather than single event or gene aberration. Thus, in the present study we sought to identify, for the first time, global major gene expressions in the most affected brain area in PD, the SNpc. Employing Affymetrix high density DNA microarray we identified 137 differentially expressed genes compared to aged-matched controls. The cerebellum of PD, an unaffected brain region served as control for tissue specificity. Confirmation of gene expressions was achieved by analysis with quantitative real-time PCR.

MATERIALS AND METHODS

Human brain tissue

Post-mortem human brains were from the National German Brain-Net center (Project no GA10) and the Netherlands Brain-Bank (Project no. 350). PD tissues were obtained from moderately to severe parkinsonism individuals based on the Hoehn & Yahr criteria in accordance with established guidelines (Hoehn and Yahr, 1967). All the subjects were negative for AD pathology according to Braak & Braak (Braak and Braak, 1997) (for details see Table 1). The average age for PD and control is 76.6 and 77.8 years, respectively. The average post-mortem delay (PMD, time interval from death to brain-freezing at -80°C) for PD and control is 26.2 and 19.8 hours, respectively. At autopsy, brains were dissected; one half was snap frozen in liquid nitrogen as slices and the other half was stored in neutral buffered

formalin for histopathology. Stringent criteria were used in the case selection of human brain tissues employed in these studies. Human SN and cerebellum regions were obtained from control and PD donors. All procedures were in accordance with the with consensus criteria established by the German and Netherlands Brain-Bank systems and in accordance NIH *Guide for the Care and Use of Laboratory human tissue* and were approved by the University of Würzburg Ethics committee, Würzburg, Germany.

Total RNA extraction

Total RNA was prepared with a phenol-guanidine isothiocyanate reagent (peqGOLD TriFast; PeQLabGmbH, Erlangen, Germany) (Lukiw and Bazan, 1997). RNA isolation reagents were prepared from 0.2 μM filtered diethyl pyrocarbonate (DEPC)-treated water (Fermentas Inc., Hanover, MD, USA) and used throughout the isolation procedure. Total RNA samples were spectrophotometrically scanned from 220 to 320 nm; the A260/A280 of total RNA was typically >1.9. In addition, formaldehyde agarose gel electrophoresis was conducted for quality control of total RNA. For all total RNAs extracted, the 28S/18S ratio was >1.5. Importantly, no significant differences in the spectral purity, rate of degradation, molecular size, or yield of SN and cerebellum total RNA between PD and control, were noted. Total RNAs was subjected to DNase-I digestion to get rid of genomic DNA residues and subsequently cleaned by the RNeasy Mini Kit (RNeasy Mini Kit; Qiagen Inc., Valencia, California, USA).

Array processing

All experiments were performed using Affymetrix HG-FOCUS oligonucleotide arrays, as described at //www.affymetrix.com/support/technical/datasheets/human_datasheet.pdf. Total RNA from each sample was used to prepare biotinylated target RNA, with minor modifications from the manufacturer's recommendations (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, 10 μg of mRNA was used to generate first-strand cDNA by using a T7-linked oligo(dT) primer. After second-strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics), resulting in approximately 100-fold amplification of RNA. A complete description of procedures is available at http://bioinf.picr.man.ac.uk/mbcf/downloads/GeneChip_Target_Prep_Protocol_CRUK_v_2.pdf. The

target cDNA generated from each sample was processed as per manufacturer's recommendation using an Affymetrix GeneChip Instrument System (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Briefly, spike controls were added to 15 µg fragmented cRNA before overnight hybridisation. Arrays were then washed and stained with streptavidin-phycoerythrin, before being scanned on an Affymetrix GeneChip scanner. A complete description of these procedures is available at http://bioinf.picr.man.ac.uk/mbcf/downloads/GeneChip_Hyb_Wash_Scan_Protocol_v_2_web.pdf.. Additionally, quality and amount of starting RNA was confirmed using an agarose gel. After scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches on the chip surface. BioB spike controls were found to be present on all the arrays, with BioC, BioD and CreX also present in increasing intensity.

Array and statistical analysis

Genes were filtered using Mas 5 algorithm results. One expected difficulty when working with post mortem samples is the various degrees of degradation in the RNA preparations. Therefore, cRNA originating from these samples contain more 3' ends than 5' prime ends. Since the Affymetrix array probe sets are designed so that the 3' ends are selected whenever possible (http://www.affymetrix.com/support/technical/technotes/hgu133_design_technote.pdf), the analysis of relatively high-3'content samples becomes feasible. Probe sets detected by MAS 5 as Present (P) have a signal with a p-value lower than 0.04 (determined from the probes contained in the probe set). This allows us to use present call with confidence. Samples that are partially degraded may hybridize to part of the probes in the probe sets leading to a high p-value (and detected as absent). If the probe set in at least four out of six samples was detected as present and all of the signals were greater than 20, we determined this probe set to be present. A list of 3517 probe sets representing genes with signals higher than 20 and detected as present (P) in all control samples, or with signals higher than 20 in all PD samples and detected as present in four out of the six samples was generated from the 8763 probe sets contained on the array

(supplement Table 2, http://eng.sheba.co.il/genomics). 262 probe sets differentiated between patient and control samples as determined by the Wilcoxon rank sum test (p-value< 0.05) (supplement Table 3, http://eng.sheba.co.il/genomics). Probe sets were further filtered selecting those where the ratio of the average (geomean) signals of the PD samples and the average signals of the control samples exceeded 1.5 or was lower than 0.66. The Bonferroni's correction has not been applied because it would result in loss of most of the valid hits. However the use of the aforementioned cut-offs together with the statistical test provide a high stringency analysis and the possibility to focus on defined subset of genes engaged in a common biological process(s). 69 probe sets were up regulated and 68 were down regulated.

Real-time quantitative PCR validation of results

In order to validate the micorarray results, we performed quantitative real-time RT-PCR for mRNA samples from PD and controls in the SNpc, pars reticulate (SNr) and cerebellum. Total RNA (1-0.4 mg) from each sample was reverse transcribed with random hexamer and oligodT primers using iScriptTM cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA, 170-8890). The genes were normalized to the house-keeping genes: beta actin (ACTB), ribosomal protein L13a (RPL13A), aminolevulinate delta synthase 1 (ALAS1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (QuantiTectTM Gene Expression Assay, Qiagen Inc., Valencia, CA, USA, Hs ACTB Assay 241013 & Hs GAPD Assay 241011), chosen after analysis according to the program geNorm (vs. 3.3; to be download from: http://medgen31.ugent.be/jvdesomp/genorm/) (Vandesompele et al., 2002; Schulz et al., 2004). The geNorm program determined the most stable house-keeping genes from a set of tested genes in a given cDNA sample panel and calculates a gene expression normalization factor for each tissue sample based on the geometric mean of a user-defined number of house keeping genes. Originally, we tested a total of six house-keeping genes, including ribosomal 18S and cyclophilin A, but the latter two were found not suitable.

These house-keeping genes were tested for their stability and found be the most accurate for normalization. Absence of DNA contamination was verified by amplifying the house-keeping gene ribosomal 18S, and running the reaction solution on agarose gel to confirm the absence of product. Minus RT samples tested simultaneously with experimental samples by quantitative RT-PCR with FAM consistently yielded no amplification below 35 cycles using the above protocol. Real-time PCR was performed in the iCycler iQ system (BioRad Co., Hercules, CA, USA) as described previously (Svaren et al., 2000; Ugozzoli et al., 2002). Briefly, 30-100 ng of cDNA and gene specific primers and probes produced by QuantiTect™ Custome Assay (Qiagen Inc., Valencia, CA, USA,) (Table 4) were added to QuantiTect Probe PCR Master Mix (Qiagen Inc., Valencia, CA, USA, 204343). Real-time PCR was subjected to PCR amplification (1 cycle at 95^0 C for 15 min, 30-45 cycles at 94^0 C for 15 s, annealing and detecting with FAM at 56^0C for 30 s and extension at 76^0C for 30 s). All PCR reactions were run in duplicate. The amplified transcripts were quantified using the comparative CT method analyzed with the BioRad iCycler iQ system program. Standard curves for each amplification product were generated from 10-fold dilutions of pooled cDNA amplicons, isolated from agarose gel using MinElute™ Gel Extraction Kit (Qiagen Inc., Valencia, CA, USA), to determine primer efficiency and quantization. Data was analyzed with Microsoft Excel 2000 to generate raw expression values. The differences in gene expression in the different brain regions were compared using the analysis of variance (ANOVA), the StatView software program (Stat View 5.0. software, SAS Institute Inc. Cary, NC, USA) on a PC computer.

RESULTS

Functional classification of differentially expressed genes in PD patients

DNA microarray analysis has been performed on postmortem SNpc from 6 parkinsonian patients and 6 aged-matched controls to identify alteration in gene transcription associated with the disease. We confined our analysis to genes with at least a 1.5 fold differential expression and significance level of $p<0.05$. Of a total of 3517 valid probe sets analyzed (see Materials and Methods), 137 met the criteria. A

heat map was performed depicting the relative expression levels of these 137 genes in PD and control samples (Fig.1), of which 68 were transcriptionally down-regulated (Table 5) and 69 were up-regulated (Table 6). Genes are clustered by their relative expression levels over the 12 samples. For negative control, five oligonucleotide hybridizations were performed in a non-related brain area, the cerebellum of both PD (2 samples) and control (3 samples), to assess tissue specificity of gene changes. Hierarchical clustering showed that both PD and normal cerebellum display a complete different pattern of expression (data not shown). Afterwards, the genes were classified into functional groups according to GeneOntology annotation tools (Dennis et al., 2003) (http://apps1.niaid.nih.gov/David/upload.asp). The Program allows finding regulation trends in group of genes organized according to biological processes, molecular function or cellular components, as defined by the Gene Ontology Consortium (http://www.geneontology.org). It is recognized that a given gene may be assigned to more than one function or biological pathway. This may result in overestimation of the true size of each functional group. We overcame this by assignment to each gene a single functional class. As shown in Fig. 2A, major gene down-regulations were observed in the signal transduction, protein degradation, dopaminergic transmission and metabolism, ion transport, protein modification/phosphorylation and energy pathways/glycolysis functional classes in PD. With respect to differentially up-regulated genes in PD, they clustered mainly in biological processes involving cell adhesion/cytoskeleton, extracellular matrix components, cell cycle, protein modification/phosphorylation, protein metabolism, transcription and inflammation/stress (Fig. 2B). Statistical analysis of gene categories, to identify genes that are the most over-represented or enriched was accomplished with the EASE program (Hosack et al., 2003). Such groupings increase confidence in the results when the proportion of genes that change within a functional group is significantly greater than the fraction of such genes in the whole chip. Functional classification with "Ease score" lower than 0.05 were marked as over represented. This analysis revealed that the most prominent changes in the differentially down-regulated genes occurred in proteolysis and peptidolysis (10%) biological process, with an EASE score of $p<0.02$ (Table 5, marked by an asterisk) and in molecular functions related to ion transporter and hydrolase activities ($p<0.05$, data not

presented). Within this proteolytic group we observed decreased expression of both catalytic and regulatory subunits of the UPS such as 20S proteasome subunits alpha-5 (PSMA5), alpha-3 (PSMA3) and alpha-2 (PSMA2), two subunits of the 19S regulatory complex of the 26S proteasome, the non-ATPase subunit 8 (PSMD8/Rpn12) and the ATPase subunit 4 (PSMC4/TBP7/Rpt3). PSMD8 slightly exceeded the upper limit of significance, but a clear trend of reduction was seen in the six patients (three of them>1.5 fold of control). Since PSMD8 clusters together with the other UPS components, we considered it as functionally affected in PD. On the other hand, statistical significant trends in biological processes and molecular functions with increased gene expression in PD were found for cell adhesion/cellular matrix (~15%) with an EASE score of $p<0.005$ (Table 6, marked by an asterisk) and for structural molecule and protein kinase activities ($p<0.05$, data not shown).

In parallel, an exceptionally stringent analysis was done selecting those genes that display a 1.5 fold differential expression in at least five out of the six parkinsonian SN. Each patient sample signals were compared to the mean of the control sample signals (geomean). This evaluation is of major importance, since it allows identifying variations in gene expression within each of the post-mortem tissue samples that may derive from a combination of factors such as age, cause of death, gender, post-mortem interval, severity of the disease, etc. This approach restricted the total differentially expressed genes in the PD cases to only 20, of which 8 were decreased and 12 were increased respective to the control group (Fig. 3). Importantly, of the 8 down-regulated genes, 3 (37.5 %) belong to DA neurotransmission and metabolism (Fig. 3A). These include cyclic AMP-regulated phosphoprotein (ARPP-21), solute carrier family 18 (vesicular monoamine member 2, VMAT2) and aldehyde dehydrogenase 1 family, member A1 (ALDH1A1). Two more genes are related to protein handling and degradation (S-phase kinase-associated protein 1A (p19A), SKP1A and the chaperone heat shock 70kDa protein 8, HSPA8). The last 3 genes are the SFRS protein kinase 2 (SRPK2), which participates in phosphorylation and protein modification processes, the tripartite motif-containing 36 (TRIM36) and the transmembrane protein with EGF-like and two follistatin-like domains 1 (TMEFF1), both of unknown function.

The 12 up-regulated genes (Fig. 3B) include PARVA (parvin, alpha), LGALS9 (lectin, galactoside-binding, soluble, 9 (galectin 9)), and SELPLG (selectin P ligand), belonging to the cell adhesion functional group. PENK (proenkephalin) and LRP6 (low density lipoprotein receptor-related protein 6), which are related to the cell signaling class. EGLN1 (egl nine homolog 1 (C. elegans)), EIF4G1 (eukaryotic translation initiation factor 4 gamma, 1), MAN2B1 (mannosidase, alpha, class 2B, member1) and SPHK1 (sphingosine kinase 1) are from the protein and lipid metabolism and phosphorylation categories. LOC56920 (semaphorin sem2), involved in cell development, ZSIG11 (putative secreted protein ZSIG11) and SRRM2 (a serine/arginine repetitive matrix 2), both of unknown functions.

Quantitative-real time PCR

To confirm our findings of decreased expression of one of the major genes SKP1A, playing a key role in the UPS, we conducted real-time quantitative PCR for it and for two additional genes, HSC70 and VMAT2, all of them affected in at least five out of six parkinsonian patients by a factor< 0.67. We added two more samples to the experimental groups (C7 and P7) in cases where the RNA used for the array hybridization was not adequate for the PCR. We analyzed three separate areas: the SNpc, SNr and the cerebellum. Cerebellum was chosen as an area not related to PD, for assessing specificity of gene changes. Real time PCR analysis revealed that the SN expresses extremely low mRNA levels of SKP1A in both normal and PD subjects (Fig. 4). Although not statistically significant, a clear trend of decreased expression in the SNpc of PD was observed (Fig. 4A). The expression of SKP1A was not affected in the SNr or in cerebellum of PD (Fig.4 B,C). We confirmed significant decreased gene expression of HSP8, coding for HSC70/HSC54, both in the SNpc and SNr of parkinsonian patients (Fig. 4 D,E), whereas no significant alterations were observed in cerebellum from PD and controls (Fig. 4F). HSC70/HSC54 exists in two isoforms, the truncated one lacking 153 amino acids residues in the protein binding and variable domain (Tsukahara et al., 2000). The probe sets in the Affymetrix chip can not discriminate between then, so the results from both the array hybridization and PCR analyses should represent a combined level of both types. In addition to these novel findings, our array analysis also confirmed

evidence for decreased expression of the vesicular DA transporter (VMAT2). Real-time PCR revealed a specific reduction of VMAT2 mRNA in the SNpc while the changes in the SNr were significantly less pronounced (Fig. 4 G,H). These alterations were specific for the SN since no significant difference was seen in the cerebellum between the PD and control samples (Fig. 4I).

To determine whether the reduced expressions found in both the arrays and in real-time PCR analysis was merely a reflection of a general reduction in mRNA levels in PD SN, or loss of synapses, we investigated the expression of three genes: Rab3b GTPase (Fig. 4 J,K,L) syntaxin 6 and the coatomer protein complex subunit zeta 2 (COP zeta 2) (data not presented), which are regulators of vesicle trafficking from endoplasmic reticulum to golgi, vesicle membrane docking and neuronal exocytosis of hormones and neurotransmitters (Gonzalez and Scheller, 1999; Futatsumori et al., 2000; Wendler and Tooze, 2001). Their expression did not differ between PD and control samples in the different brain areas.

The stringent analysis performed in this study is focused on selected biological meaningful pathways in PD and the substantial agreement of gene changes reported in literature and confirmed by real-time PCR analysis; make the analysis of the present results valid.

DISCUSSION

The results of this study show, for the first time, the global gene expression changes in the SNpc of post-mortem parkinsonian brains as compared to aged-matched controls. One major novel finding is the negative regulation of an essential component of protein catabolism, the SKP1A gene. Its decline was accompanied by decreased expression in various subunits of the 26S proteasome, in energy pathways and signal transduction, in parallel with a prominent increase in a number of genes with functional activities related to cell adhesion/cytoskeleton, extracellular matrix components and inflammation/stress.

Reduction in SKP1A, a component of the SCF ubiquitin ligase multiprotein complex, in parkinsonian SNpc

SKP1 is part of the Rbx family of RING proteins (Kamura et al., 1999) functioning within modular multiprotein Skp1, Cullin and a substrate-recognizing F-box protein (SCF). This unit allows the formation of multiple E3 complexes, which, in turn, are able to recognize a wide spectrum of different protein substrates. SCF complexes are modular: SKP1 can interact with several F-box proteins, which are responsible for specific target recognition, thereby providing functional diversity and increasing the repertoire of proteins processed by this complex. Humans express only one functional SKP1 isoform (Semple, 2003). Thus, the decrease in its expression observed in the present study, may constitute a rate-limiting factor and may account for the accumulation of a wide spectrum of ubiquitinated protein aggregates in brains of PD patients such as tyrosine hydroxylase, synphilin-1, α-synuclein, phosphorylated tau (Liani et al., 2004; Meredith et al., 2004; Zhang and Goodlett, 2004). Quantitative real time PCR analysis revealed that the SN expresses extremely low mRNA levels of SKP1A in both normal and PD subjects. For example, control samples display very low expression values (0.013±0.011), differing by more than two orders of magnitude from Rab3B (1.80±0.70), whose expression was not affected in SNpc of PD. It is also probable that the mRNA of SKP1A is particularly unstable and it degrades fast in post-mortem tissue. This may explain the great variation in the expression levels of the samples, although a clear trend of decreased expression in the SNpc of PD was observed. Because of the special design of the affymetrix chip, even samples that are partially degraded, such as in post-mortem, may in part hybridize to the probes in the probe sets leading to a high p-value and considered as present. Thus, the chip strategy together with the stringent analysis ($p<0.05$, decrease in five patients by a factor of at least 1.5), make this result confident. The low SKP1A expression levels may explain, at least in part, the particular susceptibility of the SNpc to different types of stresses, such as increased iron concentration, enzymatic (monoamine oxidase) and non-enzymatic (auto-oxidation) DA metabolism, abnormal protein accumulation, proteasomal inhibition and neurotoxin-induced OS. In this context, the PA28 multisubunit proteasome activator (a component of the 26S proteasome) protein levels were shown to be very low in the SNpc of both normal and idiopatic PD subjects compared to other brain areas (McNaught et al., 2003), possibly exacerbating the already compromised DA-

containing neurons to the various stress insults. The expression of SKP1A was not affected in the SNr or in cerebellum of PD. A recent study has demonstrated that Parkin can also function within a novel SCF-like complex, along with the F-box/WD repeat protein hSel-10, responsible for substrate recognition, and Cullin1, which however does not include SKP1 (Staropoli et al., 2003). According to this finding it is possible that Parkin associates with adaptor proteins other than hSel-10. Such complexes would likely display diverse substrate specificity and may explain the several targets that have been reported for Parkin (Dev et al., 2003).

Impairment in proteasome subunits

In addition to this selective decrement in SKP1A, a concomitant decrease in the 20S proteasome subunits alpha-5 (PSMA5), alpha-3 (PSMA3) and alpha-2 (PSMA2) mRNAs and in two subunits of the 19S regulatory complex of the 26S proteasome, the non-ATPase subunit 8 (PSMD8/Rpn12) and the ATPase subunit 4 (PSMC4/TBP7/Rpt3), was observed in the SN of PD brains, which may further contribute to dopaminergic neuronal damage in PD. The 19S complexes are located at one or both extremities of the 20S proteolytic core of the proteasome (Coux et al., 1996; Voges et al., 1999) and comprise at least 18 subunits (Glickman and Ciechanover, 2002). They are subdivided into two subcomplexes, the "base" and the "lid" which form the portions proximal and distal to the 20S proteolytic core respectively, and all together form the 26S complex. The lid consists of eight regulatory particle non-ATPase (Rpn) subunits, including PSMD8/Rpn12. One important function of the lid is to recognize multiubiquitinated proteins and other potential substrates of the proteasome (Glickman et al., 1998). The base contains three non-ATPases subunits (Rpn1, Rpn2 and Rpn10, this last common to both base and lid) and six putative ATPases subunits (Rpt1-6), including PSMC4/TBP7. These interact with nonnative conformations of nonubiquitinated target proteins (Glickman et al., 1998; Voges et al., 1999; Strickland et al., 2000) and one of them S6'/TBP1, was found to bind aggregated or monomeric α-synuclein (Ghee et al., 2000; Snyder et al., 2003). In the same context, it has been recently reported that PSMC4, also called regulatory proteasomal protein S6, specifically interacts with both wild type and mutant synphilin-1 (Duke et al., 2004), another presynaptic protein, associated with synaptic vesicles

(Ribeiro et al., 2002). Synphilin-1 was found associated with α-synuclein and similarly, it accumulates in the Lewy body (Wakabayashi et al., 2000). Thus, the decreased levels of PSMC4 found in this study, together with the progressive inhibition of the UPS by the slow but persistent aggregation of α-synuclein, may cause their accumulation in Lewy bodies (Jellinger, 2003; Liani et al., 2004). The observed increase in the expression of the glycosaminoclycan heparan-sulphate gene in PD samples, may possible contribute as well to fibrillation and aggregation of α-synuclein, as suggested (Cohlberg et al., 2002).

Each of the six ATPases is essential and they account for the ATP-requirement for proteolysis and for association of the 20S and 19S complexes to form the 26S complex (Ghislain et al., 1993; Gordon et al., 1993). Therefore, the decline in the expression of PSMC4/TBP7 in brains of Parkinsonian patients may contribute to decreased levels of 26S proteasome complex, abnormal accumulation of ubiquitinated proteins and reduced rates of degradation of short-lived proteins such as cyclins, which in turn may induce cell defects (for review see (Coux et al., 1996; Voges et al., 1999)). Indeed, we observed a decline in cyclin G associated kinase gene. Accumulation of cyclins and cyclin-dependent kinases, has been reported in post-mitotic neurons undergoing apoptosis (Padmanabhan et al., 1999; Copani et al., 2001) and in the MPTP model of PD (Grunblatt et al., 2001). It has been suggested that this event may represent an attempt at cell cycle re-entry (Verdaguer et al., 2002).

Our findings are in line with a previous report of decreased expression of the 20S proteasome α-subunits, but not β subunits, decreased protein expression levels of some 19S subunits and functional deficits in the 26/20S proteasome activity in the SNpc of patients with sporadic PD (McNaught et al., 2003). In this study however, the exact nature of the different subunits affected was not established, as the antibodies employed in the western blot analyses recognize sequences that are common to the various proteasome subunits and because of possible protein dimerization, leading to overestimation of the molecular weight. Thus, our study provides a wider view, revealing gene changes in the expression of specific proteasome components.

Heat shock protein chaperone HSC-70

Proteotoxic insults to cells or several stress conditions can induce up regulation of molecular chaperones aimed at protecting cells by assisting to correct folding of wild type and mutated proteins. One of such is the 70 kDa heat-shock cognate protein (Hsc70) (Zinsmaier and Bronk, 2001), a member of the heat-shock protein 70 (Hsp70) family. It is suggested that Hsc70 normally mediates cellular processes such as protein folding, refolding, assembly, disassembly and protein translocation via biological membranes. It has been shown that overexpression of Hsp70 reduces the amount of misfolded, aggregated α-synuclein species in vivo and in vitro (Klucken et al., 2004), prevents the loss of dopaminergic neurons in a model of PD in transgenic flies that express either the wild-type α-synuclein, as well as the mutant forms A30P and A53T (Auluck et al., 2002) and suppresses the degeneration associated with polyglutamine pathogenicity (Bonini, 2002). Recently, a functional polymorphism in the 5' promoter region of HSP70-1 has been reported in 274 PD patients, which may increase susceptibility to PD (Wu et al., 2004). We have found significant decreased gene expression HSP8, coding for HSC70/HSC54, both in the SNpc and SNr of five out of six parkinsonian patients, as confirmed by real-time quantitative PCR, whereas no significant alterations were observed between cerebellum from PD patients and controls, suggestive of tissue specificity. HSPA8 exists in two isoforms, the truncated one lacking 153 amino acids residues in the protein binding and variable domain. (Tsukahara et al., 2000) It is thought to function as an endogenous inhibitory regulator of Hsc70 by competing the co-chaperones (Tsukahara et al., 2000). At present, we can not differentiate which of these are more avidly affected.

In addition to the involvement of Hsc70 in vesicular and nuclear trafficking they may play a role in protein ubiquitination through recognition of unfolded or aberrant proteins and delivery to a co-chaperone, E3 ligase enzyme CHIP (carboxyl-terminus of Hsc70 interacting protein)(Murata et al., 2003). CHIP can cooperate with Hsp90 and/or Hsp70/ Hsc70 and ubiquitinate their attached misfolded substrates. Thus the Hsc70-CHIP pair represents an E3 ligase for specifically recognizing unfolded proteins presented by the chaperones. This molecular chaperone-UPS quality control system is of extreme importance in neurodegenerative diseases, since proteins that evade refolding or degradation by

the UPS form aggregates that accumulates into inclusion bodies. Indeed, molecular chaperones as well as ubiquitin and proteasome are recruited to inclusion bodies and Lewy Body (Stenoien et al., 1999; Sherman and Goldberg, 2001) indicating the attempt of the quality control system to degrade damaged proteins or prevent their removal. In fact, a number of neurodegenerative diseases appear to result from failure of the protein quality control system (Sherman and Goldberg, 2001).

Cell adhesion molecules, iron and oxidative stress

An association between genes playing essential roles in neuronal development, such as cell migration and axonal elongation as well as components of the cytoskeleton and AD, has recently been suggested (De Ferrari and Inestrosa, 2000). A similar hypothesis can be assigned to PD where abnormal phosphorylation of cytoskeleton components (e.g. neurofilaments, microtubule-associated proteins) and ubiquitination of synaptic and other proteins, are encountered within the Lewy bodies (Jellinger, 2003). Our present findings showing abnormal up-regulation of genes with structural and phosphorylative molecular functions integral to membrane and extracellular matrix (cell adhesion process), support this assumption and extend our knowledge revealing potential new gene targets for future therapeutics. During brain inflammation such as those reported in PD, AD and multiple sclerosis, the breakdown in the organization of key cell adhesion molecules and reduction in their signal transduction pathways may generate brain pathology, probably related to the recruitment of glia cells and macrophages and to the elevation in cytokines and OS. In addition, in five out of the six patients we observed a striking induction by more than 1.5 fold of EGLN1 (egl nine homolog 1) gene, a recently described proline hydroxylase enzyme belonging to the iron- and 2-oxoglutarate-dependent dioxygenase superfamily (Epstein et al., 2001). These enzymes act as key iron and oxygen sensor controlling the expression of the transcription factor hypoxia-inducible factor-1 alpha (HIF), a master regulator orchestrating the coordinated induction of an array of hypoxia-sensitive genes. The target genes of HIF are especially related to angiogenesis, cell proliferation/survival and glucose/iron metabolism (Lee et al., 2004). Upon high oxygen levels or iron overload, the EGLN hydroxylases targets HIF to proteasomal degradation. Interestingly, the free iron-induced proteasomal-mediated degradation of iron regulatory protein (IRP2)

involves also activation of 2-oxoglutarate-dependent dioxygenases and is inhibited by iron chelators (Hanson et al., 2003; Wang et al., 2004). Thus, it is possible that IRP2 is a substrate of EGLN1, which causes post-translational modification, signaling it for protein degradation. Excessive production of EGLN1 in the SNpc may lead to a fall in IRP2 and subsequent decrease in transferrin receptor (TfR) mRNA and increase in ferritin levels, both subjected to positive and negative transcriptional regulation by IRP2, respectively (Meyron-Holtz et al., 2004; Ponka, 2004) Recent studies in knock-out mice for IRP2, have revealed accumulation of iron in the striatum with substantial bradykinesia and tremor (LaVaute et al., 2001).

Increased expression of the iron and OS sensor protein may be directly responsible for the observed reduction in phosphofructokinase and also the angiogenic factor VEGF, both regulated by the HIF proteins (Minchenko et al., 2003). Phosphofructokinase is the key regulatory enzyme that controls the glucose flux through the glycolytic pathway. Similarly, VEGF activates genes involved in glucose transport and metabolism via activation of the PI3K and ras pathways. The expression of two major players in these pathways, ras homolog gene family, member B and phosphoinositide-3-kinase, catalytic, alpha polypeptide, were also down-regulated in parkinsonian SNpc. This finding support previous reports in human PD patients using positron emission tomography (PET) analysis, demonstrating a decrease in glucose uptake into the SN (Berding et al., 2001) and give a wider view of major survival pathways affected by the disease. These observations, together with the additional decrease in a number of energy pathways/glycolysis-related genes, as well as increases in iron/OS and inflammatory genes, is consistent with the hypothesis that mitochondrial dysfunction and reactive oxygen and nitrogen species contribute to the pathogenesis of PD. In line with this, a recent study linked a recessive mutation in the putative free radical sensor DJ-1 gene, with early-onset parkinsonism (Bonifati et al., 2003). To our surprise, apoptosis-related gene constituted the smallest functional class in PD samples, questioning the relevance of program cell death in the neurodegenerative cascade of events occurring in the disease. The role of apoptosis in PD is highly controversial since the evidence for it does not correlate with the

pathological findings and the rate of neurodegeneration. We cannot exclude the possibility that apoptotic changes occurred at earlier stages and they decline with disease progression.

Dopamine neurotransmission and metabolism

The disruption in these neuronal processes may be exacerbated by the observed reduction in the expression of cell signaling genes, which may be coupled to cell adhesion protein complexes and in genes belonging to the vesicle secretory pathway and dopaminergic neurotransmission and metabolism in the parkinsonian SN. In a highly stringent analysis, sought to detect crucial alterations in at least five out of the six PD patients, with 1.5 fold change in gene expression relative to the control group, we observed major decreases in the expression of 8 genes, 3 of them related to DA transmission, including the vesicular monoamine transporter VMAT2 (SCL18A2). The real-time PCR confirmatory analysis revealed a specific reduction of VMAT2 mRNA in the SNpc while the changes in the SNr were less pronounced. This finding is in agreement with previous reports on post mortem SN of controls and PD showing a marked reduction of VMAT2 mRNA in PD which was associated with a marked reduction in both dopamine transporter and VMAT2 signal per cell in the remaining pigmented neurons (Harrington et al., 1996; Brooks, 2003). These alterations were specific for the SN as no significant difference was seen in the cerebellum between the PD and control samples. Two other striking changes were related to the reduction in DA transmission and metabolism-related genes ALDH1A1 and ARPP-21 coding for aldehyde dehydrogenase (ALDH) and cAMP regulated phosphoprotein, respectively. ARPP-21 is specifically enriched in DA-innervated brain regions of the basal ganglia (e.g. caudate-putamen), and in the substantia nigra (Ouimet et al., 1989; Tsou et al., 1993). Similar to DARPP-32 (dopamine and adenosine 3':5'-monophosphate-regulated phosphoprotein-32K), ARPP-21 is activated by DA receptor D1, and thus may represent an index of functional activity of D-1 neurotransmission. As far as we know, this is the first report of decreased ARPP-21 mRNA levels in PD SN. ALDH1A1 was found to be expressed highly and specifically in DA cells of the SN and ventral tegmental area (VTA) and to be markedly reduced in SNpc dopaminergic neurons but not in those of the VTA of PD brains (Galter et al., 2003). This is in line with observation that striatal ALDH activity in 6-hydroxydopamine or electrical

induced lesion in rats (Agid et al., 1973) or cats (Duncan et al., 1972), was significantly reduced. ALDHs are involved in the degradation of DA to aldehyde derivatives (3,4dihydroxyphenylacetaldehyde and 4-hydroxy-3-methoxyphenylacetaldehyde) (Mardh and Vallee, 1986), which are then metabolized to homovanilin acid and dihydroxyphenylacetic acid and in detoxification of aldehydes which are highly reactive and neurotoxic (Hjelle and Petersen, 1983). Thus, alteration in DA transmission may alter ALDH activity and/or, conversely, changes in ALDH-mediated metabolism may affect DA levels in nerve cell bodies and terminal fields in basal ganglia and the limbic system. These proteins, in conjunction with VMAT2 may now be considered new markers for PD.

The overall decrease in the levels of these DA neurotransmission and metabolism-related genes observed in this study, may seriously compromise neurotransmitter storage and correlate with DA neuron dysfunction. This may further be exacerbated by the reduced levels of SEC22L1 mRNA, a member of the SEC22 family of early vesicle trafficking proteins from the endoplasmic reticulum to (Hay et al., 1996), and by the observed reduction in many membrane carrier transporters. Impairment of cell transport and vesicle trafficking, carrying a wide repertoire of intracellular and membrane proteins, may well account for their aggregation and cell deposition into cytoplasmic inclusions (aggresomes) and in the Lewy body. One simple hypothesis explaining our observations is that loss of DA-containing neuronal bodies and neuronal synapses may account for the reduce levels of the gene products. This assumption would predict similar gene expression changes in a vast repertoire of vesicle traffic or synaptic-related genes. However, as emerged from our microarray and real-time PCR confirmations, the expression of many genes related to these categories such as Rab3b, syntaxin 6 and COP zeta 2, was not altered in PD.

Protein modification/phosphorylation

The expression of two pre-mRNA splicing related genes was inversely and highly affected in each of the five or six PD samples compared to control brains: the SRPK2 gene coding for the SFRS protein kinase 2, was down-regulated and SRRM2 coding for serine/arginine repetitive matrix 2, was significantly increased. SRPK2 is a kinase highly expressed in brain with a predicted sequence for

specific phosphorylation of arginine/serine-rich (SR) domain-containing splicing factors, which in turn regulate RNA splicing in brain regions (Wang et al., 1998). Interestingly, SRRM2 (or SRm300), is a SR domain-containing splicing factor functioning as a co-activator within a major complex SRm 160/300, responsible for the processing of a subset of constitutively spliced pre-mRNAs (Blencowe et al., 2000). If SRMM2 is a substrate of SRPK2, then it seems plausible that the robust decline in the kinase SRPK2 and the high expression of SRMM2 in PD SNpc, leads to abnormal hypophosphorylated SRMM2 accumulation. This in turn may affect the nuclear distribution of SRm160 and/or other associated SR proteins converging eventually in a broad impairment in splicing activity.

CONCLUSION

In spite of the vast information regarding the pathology and etiology of PD, it is still premature to assert what is the primary event(s) that triggers the development of PD. The current study shows a reduction in the levels of selective components of the UPS together with a progressive misregulation of extracellular matrix/cytoskeleton components, concurrent with a state of OS and inflammation. These series of events may act independently or cooperatively during the course of the disease, leading eventually to the demise of dopaminergic neurons. Thus, subtle alterations in the kinetics of the different affected proteins may have, during the decades, a cumulative effect underlying the slowly progressive neurodegeneration of the DA-containing neurons in PD.

Most neurochemical and gene expression studies, including the present, on the mechanism of DA neuron death in PD and its animal models, have been conducted at the time when the majority of dopamine neurons are dead. However, more important is the detailed profile of the crucial initial neurochemical and gene expression changes in injured (but not dead) neurons, since the early gene and biochemical alterations maybe different from those at the time of neuronal death.

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CAPTURE TO FIGURES

Figure 1. Relative expression levels of the 137 genes differentially expressed in PD samples relative to control samples. Only genes that met the criteria of being altered by a factor of 1.5 relative to control and passed the Wilcoxon test at the significant level of $p<0.05$ were included. Genes are clustered by their relative expression levels over the 12 samples. Expression levels are color coded relative to the mean: green for values less than the mean and red for values greater than the mean.

Figure 2. Functional cluster analysis of genes involved in biological processes categorized according to Gene Ontology. Pie chart showing the distribution of down-regulated (A) and up-regulated (B) genes in PD samples compared to control brains. The number of altered genes in the different functional groups is indicated. Each gene was assigned a single category to avoid overestimation of the true size of each functional group. The total number of genes in (A) is 68 and in (B) is 69

Figure 3. Heat map of differentially expressed genes that changed in at least five out of six PD samples by a factor equal or over 1.5. Patient sample signals were compared to the average of the control sample signals (geomean). Down-regulated (A) and up-regulated (B) genes in PD samples compared to control brains. Expression levels are color coded relative to the mean: green for values less than the mean and red for values greater than the mean.

Figure 4. Real-time quantitative PCR analysis confirmation of differentially expressed genes in PD. Following oligonucleotide hybridization, a selected number of genes whose expression was altered in PD SN, were validated in three separate brain areas, SNpc, SNr and the cerebellum, to detect tissue specific gene alterations. For SKP1A the values were adjusted to be higher than one and a log Y axis was applied. Standard curves represent relative gene expression normalized to the geometric mean of four house-keeping genes, as described in the Materials and Methods. Data points corresponding to PD and control cases are indicated. ANOVA, $^{*}p<0.05$ vs. control

Figure 5. Schematic diagram of major gene and neurochemical alterations in SNpc of PD. Genomic analysis of SNPC of PD has confirmed and extended the previously established complexities by which dopamine neurons degenerate. These findings do not allow a conclusion to be reached regarding the primary biochemical event(s) that induces the "domino" death cascade. Oxidative stress resulting from excessive generation of nitric oxide/peroxynitrite ($NO/ONOO^{-}$), O_2 or hydroxyl radical, can lead to misregulation of iron metabolism, induction of α–synuclein aggregation and mitochondrial dysfunction. Free (labile) iron itself can cause oxidative stress, aggregation of α–synuclein and degradation of iron regulatory protein 2 (IRP2) via activation of egl nine homolog 1 (C. elegans) (EGLN1), which is a key iron and oxidative-stress (OS) sensor. This in turn, results in proteasomal degradation of hypoxia-inducible factor (HIF) and IRP2, with subsequent decreases in cell survival/proliferation, glucose and iron metabolism genes. Increase in the expression of cell adhesion molecules and components of the extra cellular matrix in response to OS /free radicals, can result in cell assembly disruption. Aldehyde derivatives of dopamine metabolism are highly neurotoxic and aldehyde dehydogenase (ALDH) is the key enzyme for their metabolism to inert acidic metabolites (homovanilic acid and dihydroxyphenylacetic acid). Gene expression of ALDH1A1, ARPP-21 and VMAT2, which are located within DA neurons of SNpc were down regulated. They may now be considered new markers for PD. Significant evidence has been provided for involvement of protein misfolding in DA neuron death. SKP1A is part of the SCF (SKP, Cullin, F-box protein) ubiquitin ligase component (E3) that regulates normal degradation of wide arrays of proteins, which may include α–synuclein, parkin, IRP2, HIF etc.

Its decline can cause evasion of proteins subjected to SCF-dependent, 26S proteasome complex degradation. This protein processing is exacerbated if some of the 26S proteasome subunits are down regulated, as observed in the present study, since they are integral part of the regulatory and catalytic activity of the proteasome. The decreased expression of the chaperone HSC-70 may affect the correct folding of several proteins that are specifically ubiquitinated by the co-chaperone carboxyl-terminus of Hsc70 interacting protein (CHIP), as well as Parkin-CHIP mediated ubiquitination (Imai et al., 2002) and may increase aggregation of α–synuclein and iron induced-OS. Red boxes are for up-regulated genes and blue boxes for down-regulated genes. Sharp arrows indicate positive inputs, whereas blunt arrows are for inhibitory inputs.

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Table 1: Summary of case selection of SNpc, SNr and cerebellum regions for the Gene Chip array and/or quantitative real-time RT-PCR analysis.

Case Number	Braak & Braak / Hoehn & Yahr	Age	Sex	PMD (Hr)	Cause of death
P1	H&Y 3/4	73	F	19	Parkinson/ renal insufficiency
P2	H&Y 5/6	79	F	17	Parkinson/ heart failure
P3	H&Y 3/4	87	F	48	n.a.
P4	H&Y 3/4	75	M	24	Parkinson/ Pneumonia
P5	H&Y 5; B&B II	78	F	29	Parkinson/ heart failure
P6	H&Y 3	70	M	22	Global heart failure
P7	H&Y 5	65	M	68	Parkinson/ Global heart failure
C1		72	M	23	ventricular fibrillation
C2		86	F	20	renal insufficiency
C3		75	M	21	Toxic heart failure
C4		85	M	20.5	Left side heart failure
C5		88	M	48	acute renal failure
C6		68	M	24	n.a.

C7		85	M	25	heart failure

Post-Mortem delay, PMD; Not available, n.a.

To see **Tables 2 and 3** go to supplements in: **http://eng.sheba.co.il/genomics**

Table 4: Sequences of oligonucleotide primer pairs and probes labelled with FAM used for real-time quantitative PCR

Gene (GeneBank accession number)	Primer pairs sequence (5' →3')	Probe sequence (5' →3'))	Product size (bp)	Cycle No.	Reaction Efficiency (%)
18s ribosomal (V01270)	n.a. (QuantiTect Hs_RRN18S Assay)		150	36	95.0
ACTB (NM_001101)	n.a. (QuantiTect Hs_ACTB Assay)		150	40	87.9
ALAS1 (NM_000688)	n.a. (QuantiTect Hs_ALAS1 Assay)		100	40	80.7
GAPDH (NM_002046)	n.a. (QuantiTect Hs_GAPD Assay)		130	35	89.0
RPL13A (NM_012423)	n.a. (QuantiTect Hs_RPL13A Assay)		100	35	99.5
VMAT2 (AI269290)	CTG TAT GTT CTT TGT TCT GGT AGA T*A GCT *T* GT* ACT TGG G	TTC CAG TCT TGC TAA CC	119	45	88.3
HSP 70kDa (AB034951.1)	TGC AGT TGG TAT TGA TCT TGG TAG CTT GGA GTG GTT CGG TT	CTC TTG TGT GG* GTG TT	107	36	97.5
RAB3B (BC005035.1)	GGGACA ATG CAC AAG TTA T AAT CAA ACC CAA GCT GCT CT	TGG AGG AAG AGA GG* GT	107	40	99.6

SKP1A (NM_006930.1)	CAG CAG GGC AGA ATA AAA AC GAGA AGA ACA GAT GGA AA	GG* GAG GCA AAG AAA GGA A	99	40	99.5
Syntaxin6 (NM_005819.1)	GTG GTG AAA GGA GAG GTA TTG GTG GTC CAG TCG ATT	CAC AGC AAC AAG G*G AAG	118	36	99.9
COP zeta 2 (NM_016429.1)	TTG ACT GAA CAG AGT GTG G GAT TGG *GG AAA TGG TCT GG	TGA AAT GAA GGC TGT GG	118	40	98.7

* Indicates modified nucleotide

n.a. not Available

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Table 5: Down regulated genes in SNpc of Pakinson's Disease

GENE BANK	SYMBOL	GENENAME	FOLD OF CONTROL	GENE BANK	SYMBOL	GENENAME	FOLD OF CONTROL
		* *Proteolysis and Peptidolysis*				*Lipid Biosynthesis*	
NM_006503.1	PSMC4	proteasome (prosome, macropain) 26S subunit, ATPase, 4	0.52	NM_002979.1	SCP2	sterol carrier protein 2	0.58
NM_002790.1	PSMA5	proteasome (prosome, macropain) subunit, alpha type, 5	0.47	BC004100.1	PIGH	phosphatidylinositol glycan, class H	0.44
NM_002787.1	PSMA2	proteasome (prosome, macropain) subunit, alpha type, 2	0.65			*Nucleobase, Nucleoside, Nucleotide and Nucleic Acid Metabolism*	
NM_002788.1	PSMA3	proteasome (prosome, macropain) subunit, alpha type, 3	0.42	BC000422.1	ARIH2	ariadne homolog 2 (Drosophila)	0.61
NM_005339.2	HIP2	huntingtin interacting protein 2	0.54	NM_016576.1	GMPR2	guanosine monophosphate reductase 2	0.47
NM_002570.1	PACE4	paired basic amino acid cleaving system 4	0.59			*Phosphate Metabolism*	
NM_006930.1	SKP1A	S-phase kinase-associated protein 1A (p19A)	0.51	NM_021129.1	PP	pyrophosphatase (inorganic)	0.56
		Dopaminergic Transmission/Metabolism				*Protein Modification/Phosphorylation*	
NM_000689.1	ALDH1A1	aldehyde dehydrogenase 1 family, member A1	0.35	NM_003640.1	IKBKAP	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	0.65
NM_016300.1	ARPP-21	cyclic AMP-regulated phosphoprotein, 21 kD	0.51	NM_002731.1	PRKACB	protein kinase, cAMP-dependent, catalytic, beta	0.57
		Energy Pathways/Glycolysis		NM_002847.1	PTPRN2	protein tyrosine phosphatase, receptor type, N polypeptide 2	0.55
NM_004373.1	COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	0.56	NM_003138.1	SRPK2	SFRS protein kinase 2	0.46
NM_002627.1	PFKP	phosphofructokinase, platelet	0.66			*RNA Processing*	
NM_000436.1	OXCT	3-oxoacid CoA transferase	0.57	NM_005872.1	BCAS2	breast carcinoma amplified sequence 2	0.58
NM_000158.1	GBE1	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme, Andersen disease, glycogen storage disease type IV)	0.45	NM_013417.1	IARS	isoleucine-tRNA synthetase	0.36
AV727381	UQCRC2	ubiquinol-cytochrome c reductase core protein II	0.59	NM_002713.1	PPP1R8	protein phosphatase 1, regulatory (inhibitor) subunit 8	0.51
		Signal Transduction				*Others and Unknown Function*	
NM_006055.1	LANCL1	LanC lantibiotic synthetase component C-like 1 (bacterial)	0.57	NM_004261.1	SEP15	15 kDa selenoprotein	0.62
NM_004238.1	TRIP15	thyroid receptor interacting protein 15	0.63	NM_003187.1	TAF9	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	0.66
NM_006218.1	PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide	0.50	NM_005667.1	ZFP103	neuroendocrine differentiation factor	0.62
NM_006226.1	PLCL1	phospholipase C-like 1	0.48	NM_004627.1	WRB	tryptophan rich basic protein	0.60
NM_005274.1	GNG5	guanine nucleotide binding protein (G protein), gamma 5	0.66	NM_014255.1	TMEM4	transmembrane protein 4	0.60
AL049933.1	GNAI1	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	0.64	AI760760	SMARCA3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 3	0.66
AF022375.1	VEGF	vascular endothelial growth factor	0.49	AI743037	FMR1	fragile X mental retardation 1	0.66
AI263909	RHOB	ras homolog gene family, member B	0.61	NM_002601.1	PDE6D	phosphodiesterase 6D, cGMP-specific, rod, delta	0.64
S77154.1	NR4A2	nuclear receptor subfamily 4, group A, member 2	0.60	NM_003919.1	SGCE	sarcoglycan, epsilon	0.63
		Transport		NM_001698.1	AUH	AU RNA binding protein/enoyl-Coenzyme A hydratase	0.60
NM_001860.1	SLC31A2	solute carrier family 31 (copper transporters), member 2	0.62	BF439316	TMEFF1	transmembrane protein with EGF-like and two follistatin-like domains 1	0.54

NM_004731.1	SLC16A7	solute carrier family 16 (monocarboxylic acid transporters), member 7	0.46	NM_000049.1	ASPA	aspartoacylase (aminoacylase 2, Canavan disease)	0.56
BC004443.1	ATP6V1E1	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E isoform 1	0.59	AL136939.1	HELO1	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	0.60
NM_005827.1	UGTREL1	solute carrier family 35, member B1	0.42	M30471.1	ADH5	alcohol dehydrogenase 5 (class III), chi polypeptide	0.62
AA890010	SEC22L1	SEC22 vesicle trafficking protein-like 1 (S. cerevisiae)	0.55	AF151522.1	C3orf4	chromosome 3 open reading frame 4	0.47
		Cell Adhesion		BC002511.1	CBR1	carbonyl reductase 1	0.51
NM_001769.1	CD9	CD9 antigen (p24)	0.39	AI984005	XPOT	exportin, tRNA (nuclear export receptor for tRNAs)	0.49
NM_021153.1	CDH19	cadherin 19, type 2	0.50	NM_016139.1	LOC51142	phosphorylase kinase, gamma 1 (muscle)	0.66
		Cell Cycle		NM_016121.1	NY-REN-45	potassium channel tetramerisation domain containing 3	0.50
NM_004417.2	DUSP1	dual specificity phosphatase 1	0.57	NM_022118.1	SE70-2	chromosome 13 open reading frame 10	0.66
BC004421.1	HSA6591	zinc finger protein 330	0.63	NM_018700.1	TRIM36	tripartite motif-containing 36	0.42
		Cell Motility/Cytoskeleton					
NM_005721.2	ACTR3	ARP3 actin-related protein 3 homolog (yeast)	0.44				
NM_004520.1	KIF2	kinesin heavy chain member 2	0.52				
BC004188.1	TUBB2	tubulin, beta, 2	0.62				

Genes are clustered into groups by biological function. The mean fold-change of each gene in PD samples relative to control samples as well as the gene symbol and GenBank accession number are indicated.

*specific categories determined to be significantly over-represented using the statistical clustering program EASE, are indicated

Table 6: Up regulated genes in SNpc of Pakinson's Disease

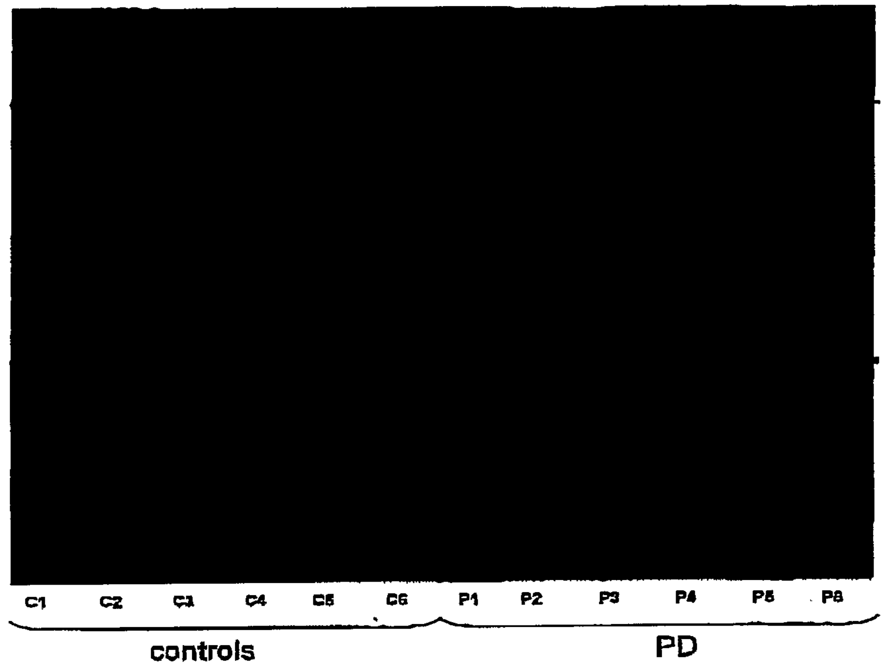
GENBANK	SYMBOL	GENE	FOLD CHANGE
		***Cell Adhesion**	
NM_014288.1	ITGB3BP	integrin beta 3 binding protein (beta3-endonexin)	1.67
NM_000632.2	ITGAM	integrin, alpha M (complement component receptor 3, alpha; also known as CD11b (p170), macrophage antigen alpha polypeptide)	1.54
AF018081.1	COL18A1	collagen, type XVIII, alpha 1	1.57
AI741056	SELPLG	selectin P ligand	2.10
AF065389.1	TM4SF9	transmembrane 4 superfamily member 9	1.60
AK022316.1	PARVA	parvin, alpha	1.80
X79683.1	LAMB2	laminin, beta 2 (laminin S)	1.78
NM_006043.1	HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	1.84
U58766	TSTA3	tissue specific transplantation antigen P35B	1.60
AI984221	COL5A3	collagen, type V, alpha 3	1.68
		Cell Motility/Cytoskeleton	
NM_002579.1	PALM	paralemmin	1.59
NM_003803.1	MYOM1	myomesin 1 (skelemin) 185kDa	2.13
M62994.1	FLNB	filamin B, beta (actin binding protein 278)	1.75
		Development	
NM_000190.1	HMBS	hydroxymethylbilane synthase	1.85
NM_000423.1	KRT2A	keratin 2A (epidermal ichthyosis bullosa of Siemens)	2.04
NM_020163.1	LOC56920	semaphorin sem2	1.98
		Cell Cycle	
NM_004383.1	CSK	c-src tyrosine kinase	1.59
AF241788.1	NUDC	nuclear distribution gene C homolog (A. nidulans)	1.52
NM_007076.1	HYPE	Huntingtin interacting protein E	1.54
D88435	GAK	cyclin G associated kinase	1.54
		Immune Response	
AI743792	SIAT1	sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase)	1.79
NM_005211.1	CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	1.57
AI073984	ICSBP1	interferon consensus sequence binding protein 1	1.88
X59350	CD22	CD22 antigen	1.60
		Response to Stress	
NM_001983.1	ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	1.81
AK023253.1	DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	1.73
		Apoptosis	
AI721219	TRAF3	TNF receptor-associated factor 3	1.63

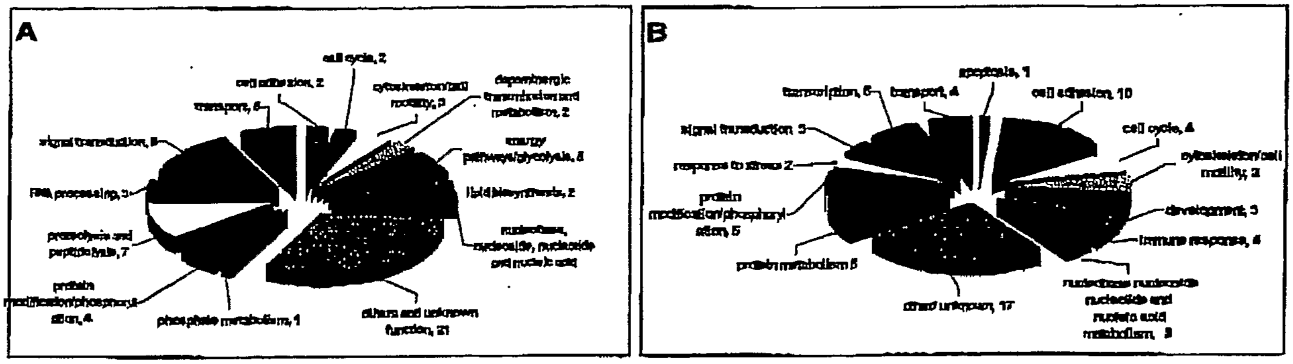
GENBANK	SYMBOL	GENE	FOLD CHANGE
		Protein Biosynthesis	
NM_004994.1	MMP9	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	2.09
BE968878	EIF4G1	eukaryotic translation initiation factor 4 gamma, 1	1.69
BC005057.1	EIF4EBP2	eukaryotic translation initiation factor 4E binding protein 2	1.75
NM_015414.1	RPL36	ribosomal protein L36	1.87
BC005369.1	EGLN1	egl nine homolog 1 (C. elegans)	2.37
		Protein Modification/Phosphorylation	
AW082913	SRPK1	SFRS protein kinase 1	1.53
AL530441	CSNK1G2	casein kinase 1, gamma 2	1.58
NM_002953.1	RPS6KA1	ribosomal protein S6 kinase, 90kDa, polypeptide 1	1.67
U68567.1	MAN2B1	mannosidase, alpha, class 2B, member 1	2.09
AF181985.1	JIK	STE20-like kinase	1.66
		Signal Transduction	
NM_005475.1	LNK	lymphocyte adaptor protein	1.51
NM_005541.1	INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	1.53
NM_006211.1	PENK	proenkephalin	2.22
		Transport	
AW167713	TCOF1	Treacher Collins-Franceschetti syndrome 1	1.71
BC001889.1	NAPG	N-ethylmaleimide-sensitive factor attachment protein, gamma	1.62
BC003068.1	SLC19A1	solute carrier family 19 (folate transporter), member 1	1.59
AF084243	ITSN1	intersectin 1 (SH3 domain protein)	1.56
		Other/Unknown	
NM_015853.1	LOC51035	ORF	1.57
NM_006556.1	PMVK	phosphomevalonate kinase	1.54
NM_004928.1	C21orf2	chromosome 21 open reading frame 2	1.83
NM_016938.1	EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	1.93
AI655799	SRRM2	serine/arginine repetitive matrix 2	1.82
AV753028	TBL1X	transducin (beta)-like 1X-linked	1.65
AA927724	APRT	adenine phosphoribosyltransferase	1.51
AA679297	SPUF	secreted protein of unknown function	1.70
NM_015710.1	GLTSCR2	glioma tumor suppressor candidate region gene 2	1.60
NM_022371.1	ADIR	torsin family 3, member A	1.52
NM_015926.1	ZSIG11	putative secreted protein ZSIG11	1.79
NM_013385.2	PSCD4	pleckstrin homology, Sec7 and coiled-coil domains 4	2.05

		Nucleobase, Nucleoside, Nucleotide and Nucleic Acid Metabolism	
NM_004349.1	CBFA2T1	core-binding factor, runt domain, alpha subunit 2; translocated to, 1; cyclin D-related	1.59
N25915	CUGBP1	CUG triplet repeat, RNA binding protein 1	1.52
AA887083	ING4	inhibitor of growth family, member 4	1.52
		Transcription	
BC004973.1	STAT6	signal transducer and activator of transcription 6, interleukin-4 induced	1.82
NM_005674.1	ZNF239	zinc finger protein 239	1.56
NM_003189.1	TAL1	T-cell acute lymphocytic leukemia 1	1.67
AF118094.1	TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	1.59
AK024501.1	MXD4	MAX dimerization protein 4	1.82
NM_005771.1	RDHL	dehydrogenase/reductase (SDR family) member 9	1.81
NM_016202.1	LOC51157	zinc finger protein 580	1.87
AF074264	LRP6	low density lipoprotein receptor-related protein 6	2.10
AC005943	MBD3	methyl-CpG binding domain protein 3	1.66
AL042496	C9orf7	chromosome 9 open reading frame 7	1.70

*specific categories determined to be significantly over-represented using the statistical clustering program EASE, are indicated

Figure 1




A
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function, 21
B
apoptosis, 1
transport, 4
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cell cycle, 4
signal transduction 5
protein metabolism 5
development, 3

Figure 2

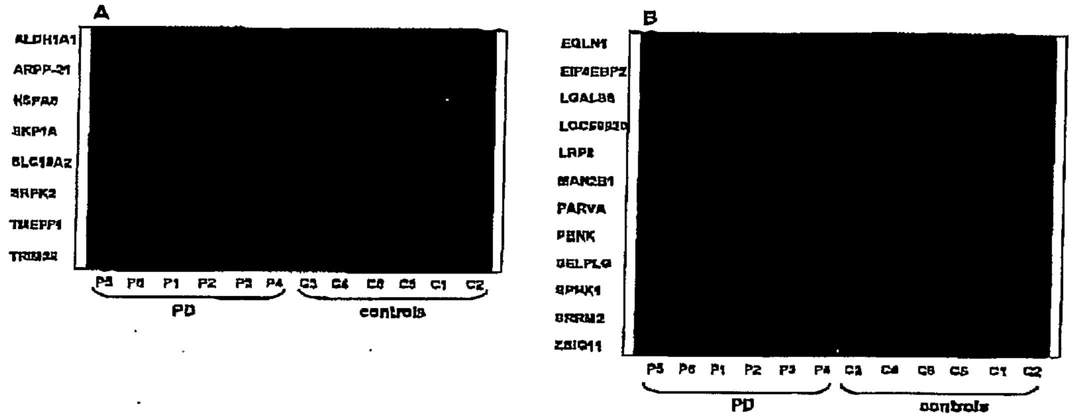

Figure 3

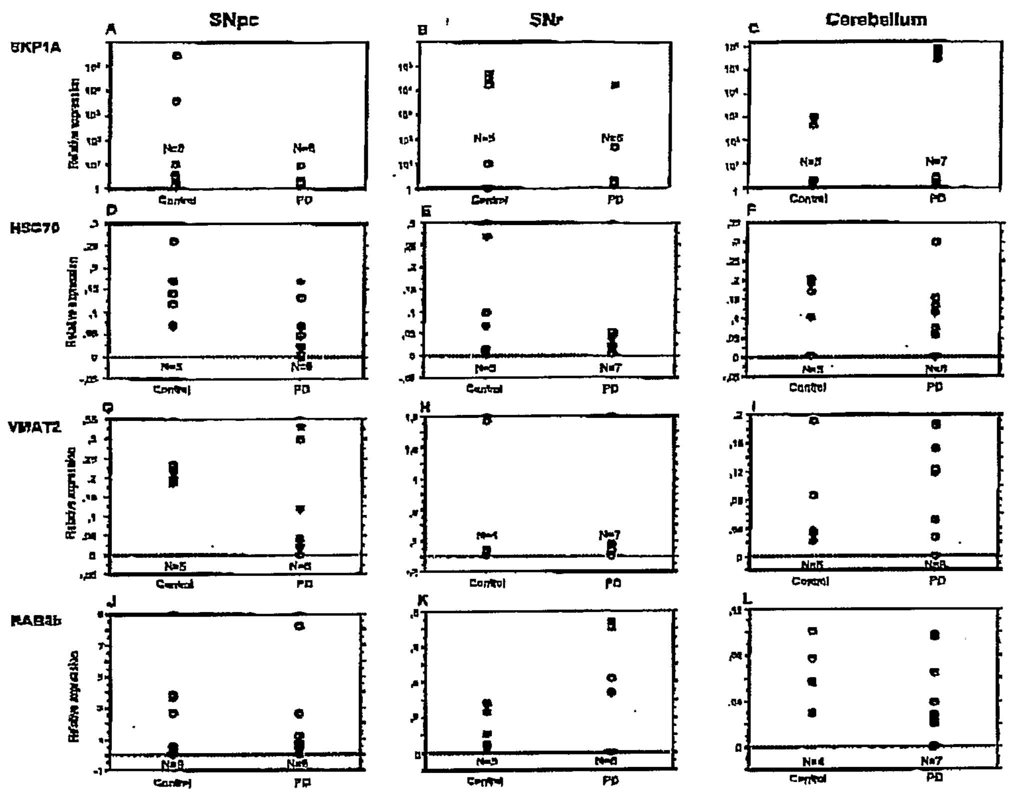
SNpc
SNr
Cerebellum
SKP1A
HSC70
VMAT2
RAB3b
Relative expression
Control
PD

Figure 4

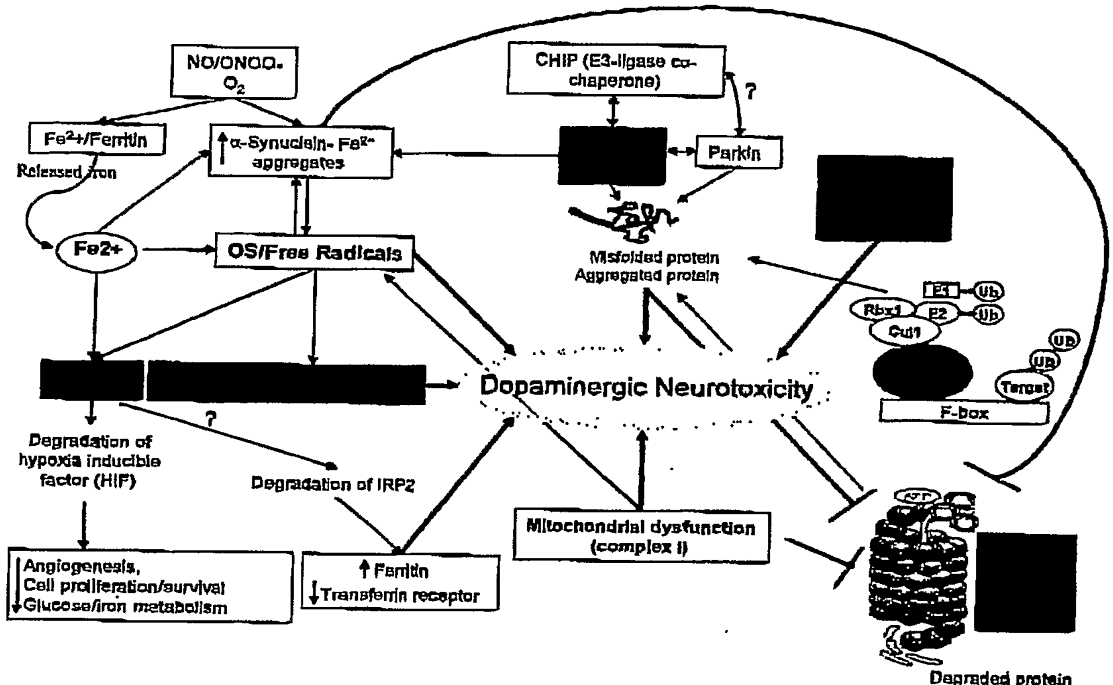
NO/ONOO. O_2
CHIP (E3-ligase co-chaperone)
?
Fe^{2+}/Ferritin
α-Synuclein- Fe^{2+} aggregates
[redacted]
Parkin
Released iron
[redacted]
Fe2+
OS/Free Radicals
Misfolded protein
Aggregated protein
E1
Ub
Rbx1
E2
Ub
Cul1
Ub
Ub
Target
F-box
[redacted]
Dopaminergic Neurotoxicity
?
Degradation of hypoxia inducible factor (HIF)
Degradation of IRP2
Mitochondrial dysfunction (complex I)
Angiogenesis,
Cell proliferation/survival
Glucose/iron metabolism
Ferritin
Transferrin receptor
[redacted]
Degraded protein

Figure 5

Claims

1. Use of molecular markers for Parkinson's disease, whereby the markers comprise genes described in table 5 and table 6.

2. Use of molecular markers for detection of Parkinson's disease, whereby the markers comprise polypeptides expressed by the genes in claim 1.

3. Use of molecular markers for detection of Parkinson's disease, whereby the markers comprise proteins and derivatives thereof expressed by the genes in claim 1.

4. A method for using molecular markers single or in combination (claim 1-3) to detect Parkinson disease.

5. A diagnosis test for Parkinson's disease comprising molecular markers (claim 1-3) single or in combination

ABSTRACT

Gene profiling of human substantia nigra pars compacta (SNpc) from Parkinson's disease (PD), was examined employing high density microarrays. We identified alterations in the expression of 137 genes, with 68 down regulated and 69 up regulated. The down regulated genes belong to signal transduction, protein degradation (e.g. ubiquitin-proteasome subunits), dopaminergic transmission/metabolism, ion transport, protein modification/phosphorylation and energy pathways/glycolysis functional classes. Up-regulated genes, clustered mainly in biological processes involving cell adhesion/cytoskeleton, extracellular matrix components, cell cycle, protein modification/phosphorylation, protein metabolism, transcription and inflammation/stress (e.g. key iron and oxygen sensor EGLN1). One major finding in the present study is the particular decreased expression of SKP1A, a member of the SCF (E3) ligase complex specifically in the substantia nigra (SN) of sporadic parkinsonian patients, which may lead to a wide impairment in the function of an entire repertoire of proteins subjected to regulatory ubiquitination. These findings reveal novel players in the neurodegenerative scenario and provide potential targets for the development of novel drug compounds.

Key words: Affymetrix, Parkinson's disease, substantia nigra pars compacta, cerebellum, gene expression, quantitative real-time PCR, ubiquitin-proteasome system, SKP1A, HSC70, iron.